Master's Thesis

The angiotensin receptor 2 agonist, compound 21, facilitates TRKB activation and reduces the consequences of stress in mice

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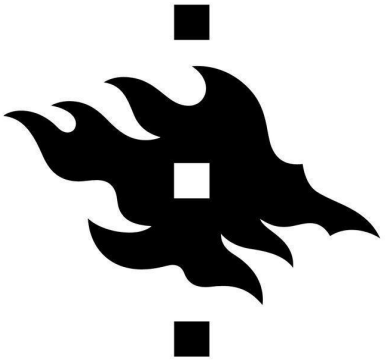

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Tiivistelmä – Referat – Abstract

This study investigated the *in vitro* and *in vivo* effects of direct angiotensin II (ANG) receptor type 2 (AGTR2) agonist Compound 21 (C21). The blockade of ANG receptor type 1 (AGTR1) by AGTR1 antagonists has long been associated with antidepressant and anxiolytic effects. Furthermore, it has been suggested that the therapeutic effects of the AGTR1 antagonists are partially dependent on enhancing the signaling through neuroprotective AGTR2. This suggests that as a specific AGTR2 agonist C21 could be used as a potential therapeutic tool to treat mood disorders that would greatly benefit from new effective treatments.

Brain-derived neurotrophic factor (BDNF) is a neurotrophic that binds to tropomyosin receptor kinase B (TRKB). This study aimed to test how C21 affects BDNF:TRKB signaling that has been shown to regulate the therapeutic effects of different antidepressants that act on mood disorders.

In vitro effects of C21 on BDNF:TRKB signaling were investigated with ELISA in the cortical cell cultures. Acute AGTR2 stimulation significantly elevated the amount of surface TRKB whereas a prolonged treatment of C21 for three consecutive days induced activation of TRKB. Similarly, combined treatment of C21 and a non-therapeutic treatment of BDNF induced TRKB activation, further linking the AGTR2 stimulation by this compound to the BDNF:TRKB signaling.

In vivo effects of C21 on conditioned and unconditioned fear were investigated in mice by using contextual fear conditioning and elevated plus-maze (EPM) respectively. The therapeutic effect of C21 protected mice from conditioned fear but failed to provide similar results for unconditioned fear in the EPM. Interestingly, these stress-protective effects of AGTR2 stimulation were lost in the BDNF-deficient animals.

To conclude, AGTR2 stimulation by C21 elevates the amount of surface TRKB that enhances the BDNF:TRKB signaling similar to antidepressants, which further leads to the therapeutic, stress-protective effects. Furthermore, these AGTR2-induced effects were absent without exposure to stress or when BDNF was diminished, indicating that both fear conditioning and BDNF are crucially involved. This study suggests that the AGTR2 is indeed a potential therapeutic target for treating mood disorders, and that in the future C21 could be translated for this use. To achieve this result, the cell types that regulate this effect need to be identified.

Avainsanat – Nyckelord – Keywords
Compound 21 (C21), angiotensin receptor type 2 (AGTR2), angiotensin receptor agonist, stress, brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TRKB)

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List of Abbreviations

AB	Antibody
ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitor
ACTH	Adrenocorticotropic hormone
AD	Antidepressant
AGTR1	Angiotensin receptor type 1
AGTR2	Angiotensin receptor type 2
ANG	Angiotensin II
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BDNF.het	Mice haploinsufficient to BDNF
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
C21	Compound 21
CNS	Central nervous system
CO_2	Carbon dioxide
CRF1	Corticotropin-releasing factor receptor 1
CRH	Corticotropin-releasing hormone
ctrl	Control
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
E18	Embryonic day 18
EAE	Enclosed arm entries
EAs	Enclosed arms (of the elevated plus-maze)
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus-maze
FBS	Fetal bovine serum
FST	Forced swimming test
GABA	Gamma-aminobutyric acid
GABAAα1	Gamma-aminobutyric acid A α1

GPCR	G-protein-coupled receptors
h	Hour
HC	Hippocampus
HPA axis	Hypothalamic-pituitary-adrenal axis
igG	Immunoglobulin G
iPlasticity	Juvenile-like plasticity
IT	Intratelencephalic
JAK-STAT	Janus kinase - signal transducer and activator of transcription
kg	Kilogram
Lamp5	Lysosomal Associated Membrane Protein Family Member 5
LH	Learned helplessness
LSD	Least Significant Difference
MANOVA	Multiple analysis of variance
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
Meis2	Meis homeobox 2
mg	Milligram
min	Minute
MKP	MAPK phosphatase
ml	Milliliter
mM	Millimolar
mPFC	Medial prefrontal cortex
N2B	Nose-to-brain route
ng	Nanogram
NP	Neuropil
OAE	Open arm entries
OAs	Open arms (of the elevated plus-maze)
OAT	Open arm time
ON	Overnight
PBS	Phosphate-buffered saline
PBS-T	PBS with 0.1% Tween
PFA	Paraformaldehyde
PFC	Prefrontal cortex

PLL	Poly-l-lysine
PPAR-y	Peroxisome proliferator-activated receptor gamma
Pvalb	Parvalbumin
PVN	Paraventricular nucleus
PV+	Parvalbumin positive
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RT	Room temperature
s	Second
sACC	Subgenual anterior cingulate cortex
Serpinf1	Serpin Family F Member 1
SFK	Src family kinase
SMC	Structural Maintenance of Chromosomes
Sncg	Synuclein-gamma
SST/*Sst*	Somatostatin
SST+	Somatostatin positive
sTRKB	Surface TRKB
TRKA	Tropomyosin receptor kinase A
TRKB	Tropomyosin receptor kinase B
TRKB:pY	Phosphorylated TRKB
UCMS	Unpredictable chronic mild stress
Vip	Vasoactive intestinal polypeptide
VLMC	Vascular and leptomeningeal cell
WT	Wild type
µg	Microgram
µl	Microliter
µM	Micromolar
%OAE	Percentage of entries in the OAs of the EPM
%OAT	Percentage of time spent in the OAs of the EPM

1 Introduction

1.1 Angiotensin receptors as possible therapeutic targets in treatment of mood disorders

Renin-angiotensin-aldosterone system (RAAS) is one of the body's regulator systems that are dedicated to maintaining homeostasis. Main functions of the RAAS are the regulation of the liquid homeostasis and blood pressure. RAAS forms a feedback system where the first released hormone triggers secretion of other hormones, which finally leads to a physiological response that when registered, down-regulates the activity of the whole system. Renin is the first product of the RAAS and it induces the conversion of circulating angiotensinogen into angiotensin I. Following this, angiotensin-converting enzyme (ACE) converts angiotensin I into the active form of angiotensin, angiotensin II (ANG). This final product is responsible for many of the physiological effects of RAAS, such as aldosterone secretion and vasoconstriction that lead to the elevation in blood pressure.

ANG receptor type 1 (AGTR1) and ANG receptor type 2 (AGTR2) are the main targets of ANG that are expressed both in the periphery and the central nervous system (CNS) (Guimond and Gallo-Payet, 2012). In general, these G-protein-coupled receptors (GPCR) are expressed by different neuronal subtypes in the brain (de Kloet et al., 2016). ANG binding to AGTR results in different physiological outcomes that are determined by the receptor type that is activated (Guimond and Gallo-Payet, 2012). The classical effects of ANG are achieved by the activation of AGTR1, Gq-type and Gi-type of GPCR, that triggers several distinct signaling pathways, including G-proteins, tyrosine kinases and mitogen-activated protein kinases (MAPK) (Hunyady and Catt, 2006). Stimulation of AGTR1 has been associated with hypertension and the development of cardiovascular diseases by particularly triggering Janus kinase - signal transducer and activator of transcription (JAK-STAT) signaling pathway (Hunyady and Catt, 2006).

Stress has been shown to elevate the activity of AGTR1 and induce anxious behavior (Saavedra et al., 2006; Campos et al., 2020). Even though classically AGTR1 antagonists have been used to treat hypertension, different AGTR1 antagonists exhibits antidepressant- (AD) and anxiolytic-like effects, indicating that AGTR1 plays an important role modulating stress-coping behavior (Srinivasan et al., 2003; Saavedra et al., 2006; Diniz et al., 2018; Campos et al., 2020). Additionally, AGTR1 antagonist telmisartan successfully restored the production of brain-derived neurotrophic factor (BDNF) that had been diminished in response to stress (Wincewicz et al., 2016), which is a common consequence of chronic stress (Castrén, 2014). Another study demonstrated that the therapeutic effect of AGTR1 antagonists is associated with enhanced signaling between ANG

and AGTR2 and triggered Fyn pathway, which results in the enhanced BDNF:TRKB signaling (Diniz et al., 2018).

Recently, AGTR1 antagonists and ACE inhibitors (ACEI) have been investigated as candidates of compounds to increase plasticity and possibly improve neuropsychiatric conditions. Compared to AGTR1 antagonist losartan that possesses an anxiolytic-like effect on hypertension-induced anxiety in rats, the effect of ACEI enalapril were found to be weaker (Srinivasan et al., 2003). Furthermore, losartan was also effective in the normotensive animals (Srinivasan et al., 2003). Interestingly, these authors speculated that the weaker effect of enalapril might be derived from its additional reduction of the neuroprotective function of AGTR2 (Srinivasan et al., 2003).

AGTR2 is a Gi-type of GPCR and it's activation triggers, for instance, phosphatase pathways, kinase pathways, and the activation of other receptors (Guimond and Gallo-Payet, 2012). These signaling pathways include peroxisome proliferator-activated receptor gamma (PPAR-y), MAPK phosphatase (MKP), and Src family kinase (SFK), such as Fyn (Guimond and Gallo-Payet, 2012). In contrast to AGTR1, AGTR2 stimulation is assumed to be neuroprotective (Namsolleck et al., 2013) and AGTR2-deficiency leads to increased anxiety (Okuyama et al., 1999). Thus, as the therapeutic effects of AGTR1 antagonists may be dependent on facilitating the interaction between ANG:AGTR2 (Diniz et al., 2018), AGTR2 agonists, that directly enhance the function of this receptor, might offer a novel tool to treat mood disorders.

Compound 21 (C21) is the first synthesized nonpeptide molecule that selectively targets and activates AGTR2 (Wan et al., 2004). This AGTR2 agonist increases neuronal outgrowth (Wan et al., 2004; Namsolleck et al., 2013), neuronal reinnervation and axonal plasticity (Namsolleck et al., 2013). C21 has also been observed to attenuate the neuronal consequences of ischemic stroke and myocardial infarction (McCarthy et al., 2014; Bennion et al., 2018). Additionally, C21 has been associated with anti-inflammatory (Kaschina et al., 2008; Rompe et al., 2010) and anti-apoptotic effects that led to improved cell survival (Kaschina et al., 2008; Namsolleck et al., 2013). C21 has been shown to potentiate the effect of AGTR1 antagonist candesartan on the elevated blood pressure in hypertensive rats (Bosnyak et al., 2010). However, C21 by itself seems to have no antihypertensive properties (Bosnyak et al., 2010; Bennion et al., 2018).

The beneficial effects of C21 are prevented in the cells lacking AGTR2 or that had been previously exposed to the AGTR2 antagonist PD123319 (Namsolleck et al., 2013). Similarly, the neuroprotective effect of this AGTR2 agonist on stroke disappeared in the rats that were treated with the combination of C21 and PD123319 (McCarthy et al., 2014). Together these studies

confirm that the selective stimulation of AGTR2 is indeed an essential part of the neuroprotective effects of this drug. Moreover, the beneficial effects of C21 seems to involve neurotrophin signaling as the neurotrophin receptor inhibitor K252a efficiently compromises the neuronal outgrowth in the C21-treated cells (Namsolleck et al., 2013). Interestingly, an enhanced synthesis of BDNF, tropomyosin receptor kinases B (TRKB) and A (TRKA) was observed in the cells treated with C21 (Namsolleck et al., 2013).

1.2 The role of BDNF and TRKB signaling in AD-induced plasticity

TRKB is the high-affinity receptor of BDNF, and together these molecules participate in the activity-dependent plasticity that allows the CNS to adapt to the constantly changing internal and external environmental circumstances (Castrén and Antila, 2017). This process is dependent on the activity of neuronal connections, and thus the most commonly used connections are enhanced (Castrén and Antila, 2017). On the contrary, the connections that lose the competition to the more active ones perish (Castrén and Antila, 2017). The activity of neuronal connections leads to the enhanced BDNF:TRKB signaling that determines the final outcome of activity-dependent plasticity (Castrén and Antila, 2017). Furthermore, it has been widely accepted that the therapeutic effects of classical and atypical ADs work through a BDNF:TRKB-dependent mechanism of action (Castrén and Antila, 2017) and elevate the activity of TRKB in the brain (Umemori et al., 2018). Interestingly, the major depressive disorder (MDD) seems to result in the reduction of the serum BDNF (Karege et al., 2002). Additionally, BDNF-deficient animals do not benefit from the AD treatments (Karpova et al., 2011), which further demonstrates the crucial role of BDNF. Because of this, compounds that are able to enhance BDNF:TRKB signaling could be used as new therapeutic tools to treat mood disorders.

According to the network hypothesis of depression, the mood disorders are an end result of altered signaling of the neural network that can be recovered with the AD treatment (Umemori et al., 2018). By enhancing the BDNF:TRKB signaling, ADs are able to produce a critical period-like state called juvenile-like plasticity (iPlasticity), where the plasticity is elevated (Castrén and Antila, 2017; Steinzeig et al., 2017; Umemori et al., 2018; Steinzeig et al., 2019). Interestingly, iPlasticity allows the neuronal network of an adult brain to be modified more freely through activity-dependent plasticity (Castrén and Antila, 2017, Umemori et al., 2018).

Activity-dependent plasticity and the mood recovering effect of ADs are closely related to BDNF:TRKB signaling. For example, the BDNF synthesis and the amount of surface TRKB has been shown to increase in response to neuronal activity (Castrén and Antila, 2017; Umemori et al.,

2018). Furthermore, chronic AD treatments induce elevated BDNF and TRKB synthesis, whereas the acute treatments are able to enhance TRKB activation (Castrén and Antila, 2017). Direct manipulation of BDNF has provided an interesting standpoint to investigate its role in the AD effect. For example, chronic infusion of BDNF into the midbrain of rats improved their performance in the learned helplessness (LH) and forced swimming test (FST) (Siuciak et al., 1997). Furthermore, an acute infusion of BDNF into the hippocampus (HC) of rats induced a long-lasting antidepressant-like effect (Shirayama et al., 2002). Our recently published study has also shown that AD-induced plasticity and other therapeutic effects of different ADs are induced by the direct interaction between ADs and a specific motif of TRKB transmembrane domain (Casarotto et al., 2021).

Since ADs allow the reinstatement of plasticity, the best outcome of treating mood disorders is achieved when the reshaping of neuronal networks is guided by a proper activity such as psychotherapy (Castrén and Antila, 2017; Umemori et al., 2018). In fact, there is already evidence supporting this finding, whereas the ADs and psychotherapy on their own have turned out to be less effective (Pampallona et al., 2004; Umemori et al, 2018). Furthermore, the AD fluoxetine reduces the consequences of contextual fear conditioning when the fear memories are reshaped by extinction training (Karpova et al., 2011). However, the combined effects of these treatments seem to be necessary for the observed therapeutic effects as they disappear in mice that only received fluoxetine without extinction training or vice versa (Karpova et al., 2011). Interestingly, the combined treatment was not able to attenuate fear in BDNF-deficient animals (Karpova et al., 2011), and similarly the antidepressant-like effect induced by AGTR1 antagonist losartan is diminished in these animals (Diniz et al., 2018). In fact, the therapeutic effects of ADs disappear when either BDNF or TRKB function has been compromised (Castrén and Antila, 2017). All in all, it can be concluded that the BDNF:TRKB signaling plays a crucial role in the AD-induced plasticity.

1.3 Contextual fear conditioning and anxiety

Contextual fear conditioning, also known as Pavlovian fear conditioning, is a validated method to investigate fear and formation of fear memories (Giustino and Maren, 2015). In the contextual fear conditioning the animals are exposed to a distinguishable but neutral context that will be associated with a fear-inducing stimulus. When animals are returned back to the familiar context, the environment by itself should induce fear, observed as freezing, even when the fear-inducing stimulus is not present. The effects of ADs have also been investigated in the contextual fear

conditioning where their therapeutic effects lead to reduced freezing and thus, relieved conditioned fear (Burghardt and Bauer, 2013). For example, a chronic treatment of AD fluoxetine has been able to reduce the consequences of stress in rats that were exposed to fear conditioning (Zhang et al, 2000).

Fear conditioning consists of the acquisition, consolidation and retrieval of fear memories that can be attenuated to some extent by the extinction training where the animals are re-exposed to the source of fear without the fear-inducing stimulus (Burghardt and Bauer, 2013). However, extinction training by itself is not able to prevent spontaneous recovery (recovery of freezing response with re-exposure) but when combined with AD treatment, the fear memories can be overwritten by new, neutral experiences in the familiar context (Karpova et al., 2011).

The elevated plus-maze (EPM) is a validated ethological model to investigate anxiety and anxiolytic-like effects of drugs (Walf and Frye, 2007), such as ADs. In comparison to contextual fear conditioning, generally EPM does not contain any conditioning stimulus (Walf and Frye, 2007). Instead, the anxious behavior is induced in rodents by the distressing environment as the apparatus is placed above the floor and the two of the four arms of the EPM lack surrounding, protective walls (Walf and Frye, 2007). As a result, an approach-avoidance conflict towards the open arms (OAs) of the EPM is established, and in general, animals show a clear preference towards secure enclosed arms (EAs) of the apparatus (Walf and Frye, 2007). The anxiolytic effect of ADs reflect on the animal's behavior by increasing their interest towards approaching the OAs, which would lead to the elevated OA activity (Walf and Frye, 2007). However, to gain a deeper insight into the mechanisms of conditioned and unconditioned fear, it is important to understand their relation to the brain.

Fear and formation of fear memories are regulated by several different parts of the brain that together form an interconnected network, respectively a fear circuit (Giustino and Maren, 2015). The main regulators of the fear circuit are the amygdala, HC and medial prefrontal cortex (mPFC), the latter being the structure that guides the functions of the other parts of this system according to received and previous information (Giustino and Maren, 2015). Fear conditioning leads to alterations in the activity of the mPFC-located neurons even when the animals are exposed to the familiar context in the absence of fear-inducing stimulus (Baeg et al., 2001). Interestingly, the activity of these neurons is restored by extinction training (Baeg et al., 2001). Whereas mPFC regulates the behavioral aspects of fear, the acquisition of conditioned fear is modulated by the amygdala and HC, which crucially contributes to storing contextual information in the long-term memory (Giustino and Maren, 2015). Furthermore, amygdala participates in the extinction of

conditioned fear (Giustino and Maren, 2015), and in fact the beneficial effect of fluoxetine on fear extinction is based on enhancing the activity-dependent plasticity in the amygdala (Karpova et al, 2011).

Similar to conditioned fear, the fear circuit is also involved in the regulation of anxiety and unconditioned fear along with the bed nucleus of stria terminalis (BNST) located in the proximity of amygdala (Calhoon and Tye, 2015). Unconditioned fear response consists of four stages, respectively detection, identification, evaluation and finally, behavioral responses (Calhoon and Tye, 2015). The amygdala is responsible for identifying the potential threat and inducing unconditioned fear response whereas the other parts of the circuit further confirm if the situation is indeed threatening (Calhoon and Tye, 2015). Compared to conditioned fear, the fear circuit works in a rather similar manner in the unconditioned fear as the mPFC determines the final behavioral outcome of the fear response (Calhoon and Tye, 2015).

Despite the carefully structured regulation system, even harmless environmental stimuli can be identified as a threat in some circumstances, followed by the typical anxious behavior such as activation of the sympathetic nervous system and fight-or-flight response (Calhoon and Tye, 2015). Furthermore, the network signaling can be altered chronically as observed in the MDD, which has been associated with a significant downregulation of cortical gamma-aminobutyric acid (GABA) accompanied by an upregulation of glutamate (Sanacora et al., 2004). Since GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, the alterations in their abundance impact the whole network signaling (Sanacora et al., 2004).

2 Hypothesis

The administration of C21 facilitates the activation of TRKB, which in turn reduces the consequences of stress and anxiety in mice.

3 Aims of the study

This study aimed to test if the *in vitro* and the *in vivo* effects of C21 are related to the BDNF:TRKB signaling. The *in vitro* effects were investigated with enzyme-linked immunosorbent assay (ELISA) whereas contextual fear conditioning and EPM were used to address the effect of C21 in stress and anxiety. The following questions were covered in this study:

1) Does C21 treatment elevate the amount of surface TRKB?
2) Is C21 treatment able to facilitate the effect of BDNF *in vitro*?
3) Does C21 protect from the stress and anxiety *in vivo*?
4) Are these *in vivo* effects of C21 dependent on BDNF?
5) What cell subtypes express AGTR2 in the brain?

4 Material and methods

4.1 *In vitro* experiments

4.1.1 Drug treatments *in vitro*

C21 (sodium salt, MW = 497.61, purity = 97.7%) was provided by Vicore Pharma, Sweden as a kind gift. Dimethyl sulfoxide (DMSO) was used as a vehicle for C21 (ctrl; 0.1; 1.0; 10μM). Respectively, phosphate-buffered saline (PBS) was used as a vehicle for recombinant human BDNF (Peprotech, #450-02, 0.1 ng/ml).

4.1.2 Cortical cell cultures, drug treatment and sample collection

The rat cortical cell cultures were used in all *in vitro* experiments. All cell cultures were kindly prepared for this project by lab technicians of the Neuroscience Center, using embryonic day 18 (E18) rat embryos according to literature (Sahu et al., 2019). First, the cortex of rat E18 embryos were dissected, the collected tissue was triturated and the cells isolated, centrifuged and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. Eventually, after making sure all other cells and their components were removed, the cortical cells were suspended in the growing medium (2% B-27, 1% L-glutamine and 1% penicillin/streptomycin in NeuroBasal medium) and seeded in pre-coated wells.

The 96-well plates were coated with 200 μl/well of 10 μg/ml Poly-l-lysine (PLL) and similarly 500 μl/well of PLL was used with 24-well plates, after which the plates were left to incubate overnight (ON) at 37°C prior to the cell seeding. Once a week, 30% of the media was replaced by fresh media but otherwise the cells were kept in the incubator for 8-10 days *in vitro*. The cells were cultivated in 96-well plates (60 000 cells/well in 100μl/well of growing medium) for direct ELISA experiments; or in 24-well plates (250000 cells/well in 500 μl/well of growing medium) for sandwich ELISA experiments.

For the treatment, the cells that were fixed for direct ELISA received C21 (0.1; 1.0; 10μM) for 15min. The cells that were lysed for sandwich ELISA experiments received C21 (10μM) either once for 15min followed by a non-therapeutic treatment of BDNF (0.1ng/ml) for 15min; or C21 once a day for three consecutive days, similarly treated with BDNF 2h after the third treatment of C21. The same concentrations were used for prolonged treatments.

As soon as the drug treatments were conducted, the cells were washed with ice-cold PBS to get rid of the remaining substances, and either fixed or lysed depending on the experiment in question. Every time the plate was incubated for any means, it was kept on a rocking platform to

maintain stable conditions throughout the samples. For direct ELISA, the cells were fixed by leaving them in 75µl/well of 4% paraformaldehyde (PFA) for 20min at room temperature (RT), followed by blocking. On the other hand, the lysing for sandwich ELISA was carried out by exposing the cells to fresh NP+ lysis buffer (3M Tris-HCl, 5M NaCl, 0.5M NaF, 1% Nonidet-40, 100mM Na_3VO_4, 10% glycerol in MilliQ-water, supplemented with phosphatase inhibitors (Sigma-Aldrich, #P0044) and protease inhibitors (Sigma-Aldrich, #P2714)) for 30min at +4°C. When the lysing was finished, the samples were immediately added to the blocked wells for next steps of sandwich ELISA.

4.1.3 Direct ELISA

Direct ELISA was used to investigate whether C21 is able to increase the amount of surface TRKB (sTRKB) similar to previous studies (Zheng et al., 2008; Fred et al., 2019). At first, the already fixed and collected samples were carefully washed by incubating them in PBS for 5min at RT for three consecutive washings, after which 200 µl/well of blocking buffer (5% nonfat dry milk and 5% of bovine serum albumin (BSA) in PBS) was added in the samples for 1h at RT. To investigate the amount of TRKB, the primary antibody (AB) that specifically targets this receptor (R&D; #AF1494; 1:500) was dissolved in the blocking buffer, the wells were filled with 100 µl/well of the solution, and the plate was then sealed and incubated ON at +4°C.

On the second day, the plate was washed with PBS and the secondary, HRP-conjugated AB targeting goat immunoglobulin G (IgG, Invitrogen; #61-1620; 1:5000) was dissolved in the blocking buffer and 100 µl/well of the final solution was applied to the wells for 1h at RT. Next the plate went through four, similar consecutive washings in PBS at RT, changing the liquid between the washes that each took 10min from start to finish. At the end of the experiment, the wells of the plate were filled with 100 µl/well of enhanced chemiluminescence (ECL, 1:1) solution that was used to quantify the amount of sTRKB by Varioskan Flash, Thermo Scientific. The amount of sTRKB was gained when the background (wells without cells) was discounted from the chemiluminescence emitted from the samples. The untreated controls were compared to C21-treated samples, and the final results were displayed percentually.

4.1.4 Sandwich ELISA

The effect of C21 on BDNF-induced phosphorylation of TRKB (TRKB:pY) was studied with sandwich ELISA by the already established instructions (Fred et al., 2019). In the beginning of the experiment, the primary AB targeting TRKB (R&D; #AF1494; 1:1000) was dissolved in a carbonate buffer (57.4mM NaHCO3, 42.6mM Na2CO3, pH = 9.8), and a prepared solution was

used for coating the 96-well plate, 100 µl/well respectively. The plate was sealed and incubated ON at +4°C. On the second day, the plate was prepared for drug-treated samples by incubating in 200 µl/well of blocking buffer (2% BSA in PBS with 0.1% Tween 20 (PBS-T)) for 2h at RT. When the drug treatments and the sample collection were finished, the plate was returned to the +4°C room ON.

On the third day, the plate was washed with PBS-T. To measure the phosphorylation in the samples, they were exposed to 100 µl/well of biotinylated secondary AB that targets tyrosine phosphorylated proteins (BioRad; #MCA2472B; 1:2000) dissolved in the blocking buffer at +4°C ON. On day four, the plate was washed and the previous AB solution was replaced with 100 µl/well of tertiary HRP-conjugated streptavidin solution (Thermo Scientific Pierce; HRP-streptavidin; #21126; 1:5000), respectively prepared in the blocking buffer, 2h at RT. One last washing was conducted and 100 µl/well of ECL (1:1) was applied to quantify TRKB:pY in the samples by Varioskan Flash, Thermo Scientific. The results were processed and interpreted in a similar manner as in the direct ELISA.

4.2 *In vivo* experiments

4.2.1 Drug treatments *in vivo*

Sterile saline was used as a vehicle for C21 (0.3 mg/kg; 0.009 µg/day) (Namsolleck et al., 2013) and administered during isoflurane-induced (2%, Vetflurane®, Virbac) anesthesia.

4.2.2 Mouse strains

In order to investigate if BDNF is crucially involved in the effect of C21, a strain of *Bdnf* haploinsufficient (BDNF heterozygous, BDNF.het) mice was used. These animals exhibit significantly attenuated synthesis of BDNF in several brain regions (Ibarguen-Vargas et al., 2009; Hill and van den Buuse, 2011).

4.2.3 Animals

In this study, 34 female mice (C57BL/6J-000664 background) were exposed to contextual fear conditioning (20 wild types (WT) and 14 BDNF.het) and 12 for EPM (all WT). All animals were genotyped upon weaning, and were at the 16-18 week old age during the experiments.

All animals were maintained in the Laboratory Animal Center in the Viikki Campus of University of Helsinki, group-housed (4-5 animals per cage) in type ll individually ventilated cages (552 cm^2 floor area, Tecniplast, Italy). During all times outside the drug treatments and experimental procedures, the animals are provided with a constant supply of food and water. As the

final step of each behavioral experiment all animals are appropriately euthanized with CO_2, followed by cervical dislocation. All procedures used in this study were approved by the Experimental Animal Ethical Committee of Southern Finland (ESAVI/38503/2019).

4.2.4 Administration of C21

The adoption of AGTR2 agonists, such as C21, into clinical use has been limited as they generally do not penetrate blood brain barrier (BBB) and are therefore unable to access CNS at therapeutic levels (Shraim et al., 2011; Bennion et al., 2018). Similarly, C21 has trouble penetrating the BBB and generally does not reach the CNS at therapeutic levels when administered intraperitoneally or intravenously (Shraim et al., 2011; Bennion et al., 2018).

Fortunately, a solution has been found in administering these BBB-impermeable substances through the nose-to-brain route (N2B) (Dhuria et al., 2010; Bennion et al., 2018; Hanson et al., 2013), where any desired substance is directly administered in the nasal cavity of the animal where it is absorbed and delivered to the CNS via the olfactory nerves, trigeminal nerves and perivascular transport (Dhuria et al., 2010). Another advantage of this administration route lies in its specificity due to which it is possible to use lower doses whereas the systemic exposure to the administered substance remains low (Dhuria et al., 2010). In fact, this administration route has been successfully used to deliver C21 to the CNS, followed by therapeutic, neuroprotective effects (Bennion et al., 2018).

Regarding this study, N2B administration of drugs was carried out by following the protocol found in the literature (Hanson et al., 2013) with slight adjustments. Even though the administration could have been executed without anesthesia, the decision to use a mild, isoflurane-induced anesthesia was made to ensure that the animals were easily handled and less stressed by the procedure. At first, the group-caged mice were moved to the transparent chamber where they were exposed to 2% of isoflurane for 4min. Before administration, the level of anesthesia was tested by pinching the paw gently to investigate paw reflexes. The animal was held vertically, and the head was tilted, and then the N2B drug administration was carried out with the help of a micropipette attached with a long and thin tip suitable for delivery. Finally, 20µl of the assigned treatment was administered through N2B depending on the treatment group of the animal (WT/vehicle, WT/C21, BDNF.het/vehicle, BDNF.het/C21). The other nostril was used for the drug treatment the following day. The drug treatments were continued for three consecutive days, followed by either contextual fear conditioning or EPM 2h after the treatments were completed.

4.2.5 Treatment groups

In the contextual fear conditioning, there were four different groups: WT/vehicle (N = 8), WT/C21 (N = 12), BDNF.het/vehicle (N = 6) and BDNF.het/C21 (N = 8). In the EPM, there were only two treatment groups: WT/vehicle (N = 6) and WT/C21 (N=6).

4.2.6 Contextual fear conditioning

The contextual fear conditioning was used to investigate how C21 affects the response to stress (Michels et al., 2018). During the experiment, the freezing time (s), the mean velocity (cm/s) and travelled distance (cm) were recorded by TSE Instruments, Germany. Following the drug treatments (Figure 1A), one animal at the time was exposed to the Context A (23x23x35cm, chamber with clear acrylic walls and metal grid floor, Figure 1B) where three electric foot shocks (0.6mA/2s) were delivered in 10min. Shocks were assumed to induce fear conditioning, associated with this specific context. The mean velocity of each animal was measured to confirm that all animals actually received and responded to the shocks. Travelled distance was measured during the first 2min before any shocks were delivered to estimate the locomotion and activity of each animal.

On the following day, the chamber was replaced by an unfamiliar Context B (23x23x35cm, chamber with black acrylic walls and black acrylic floor, Figure 1C), and the freezing response was monitored in the absence of shocks to investigate if the conditioning induced fear in general. Animals were exposed to Context B for 5min. On the following day animals were re-exposed to the familiar Context A to investigate if the familiar environment by itself would induce freezing in the 5min (Figure 1D). In the end, the freezing times between the conditioning session and re-exposing to familiar Context A were compared with each other to investigate how different groups responded to the context-induced stress.

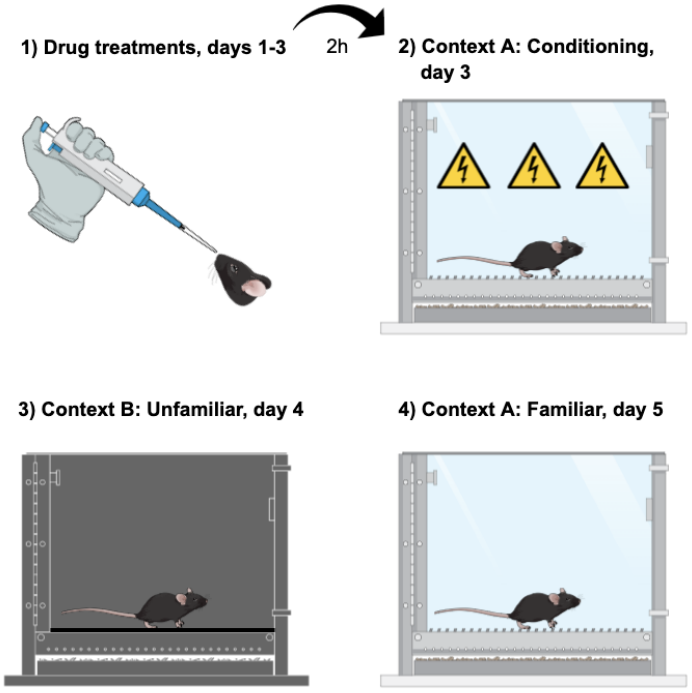

Figure 1. Experimental design of contextual fear conditioning. A) The animals were treated daily with desired drug treatments (vehicle, C21) on three consecutive days. **B)** When treatments were finished, the animals were left alone for 2h before Context A was used to induce contextual fear conditioning by electric foot shocks, three shocks in 10min. **C)** In the unfamiliar Context B, animals were investigated for generalized fear for 5min. **D)** Finally the mice were re-exposed to the familiar Context A to measure context-induced freezing in 5min.

4.2.7 Elevated plus-maze

In the present study, the EPM was used to investigate the fear and anxiety in an ethological setting without conditioning after C21 treatment. The EPM was adjusted following the general protocol found in the literature (Walf and Frye, 2007). The EPM is a plus-shaped apparatus (Figure 2A) with two OAs (30 x 5cm) and EAs (30 x 5cm) that are surrounded by the protective walls (20cm). The whole apparatus was placed 40cm from the floor, creating an ethological experience of fear.

In the beginning of the experiment, one animal at the time was placed in the middle area (5 x 5cm) of the EPM (Figure 2B). During the whole experiment (5min), the animal was recorded by Ethovision XT 13, Noldus, Netherlands, and the movement of the animal was measured as travelled distance, presenting the overall activity of the animal. The number of entries to the EAs and the percentage of entries to the OAs of the EPM were measured as enclosed arm entries (EAE) and open arm entries (%OAE). Additionally, the percentage of time animals spent in the OAs of the EPM was counted (%OAT). If the drug induced an anxiolytic effect, the OAE and OAT were assumed to increase, while any locomotor effect of the treatment should reflect on EAE and the travelled distance.

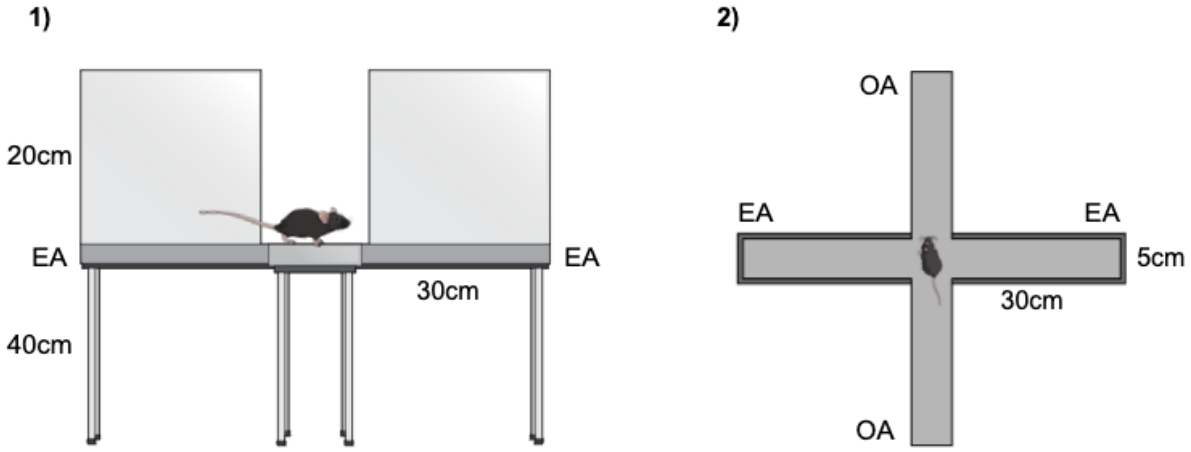

Figure 2. Experimental design of EPM. A) Picture of the EPM apparatus from the side. EPM is placed 40cm upon the floor, inducing unconditioned fear. **B)** EPM has two open arms (OAs) and enclosed arms (EAs) of similar size. The OAs of the EPM are also part of the ethological nature of this method as they create approach-avoidance conflict. The apparatus used in the present study was made of acrylic with opaque floors and transparent walls.

4.3. *In silico* experiments

To further investigate what cells might be involved in the effect of C21, we wanted to investigate the expression of AGTR2. The data from Allen Mouse Brain Atlas was used to mine information of different cell subtypes that might express this receptor in the brain (Lein et al., 2007; Morris et al., 2010). Using the genome browser tool for the mouse ribonucleic acid (RNA) sequencing data (https://celltypes.brain-map.org/rnaseq/mouse/v1-alm), a heatmap was created for the expression of *Agtr2* in different classes and subclasses of cells in the visual and motor cortices.

Cell Classes: GABAergic, glutamatergic, non-neuronal, endothelial; **Subclasses:** Lysosomal Associated Membrane Protein Family Member 5 (*Lamp5*), Synuclein-gamma (*Sncg*), Serpin Family F Member 1 (*Serpinf1*), Vasoactive intestinal polypeptide (*Vip*), Somatostatin (*Sst*), Parvalbumin (*Pvalb*), Layer 2/3 intratelencephalic (IT), Layer 4, Layer 5 IT, Layer 6 IT, Layer 5 principal neurons, Neuropil (NP), Layer 6 corticothalamic, Layer 6b, Meis homeobox 2 (*Meis2*), Calcitonin receptor, Astrocyte, Oligodendrocyte, Vascular and leptomeningeal cell (VLMC), Periostin, Structural Maintenance of Chromosomes (SMC), Endothelial, Macrophage.

4.4. Statistical Analysis

Kruskas-Wallis test and two-way ANOVA [Factors: C21 effect and BDNF effect] were used as the main analysis methods for the analysis of *in vitro* experiments. Kruskal-Wallis test indicated a

significant difference in the treatment groups that was further investigated with Dunn's *post hoc* test. Similarly, two-way ANOVA was accompanied by Fisher's Least Significant Difference (LSD).

Two-way ANOVA, a multiple analysis of variance (MANOVA) and the two-tailed unpaired t-test were used to analyse *in vivo* experiments. The contextual fear conditioning was mostly analysed with the two-way ANOVA but in order to allow an overall analysis of the interaction between treatment and genotype along the fear conditioning session (freezing in the last 2min of conditioning, in the unfamiliar Context B and in the familiar Context A), all groups (WT/vehicle; WT/C1; BDNF.het/vehicle; BDNF.het/C21) were analysed with MANOVA. MANOVA analysis was carried out with the trials as repeated measures, genotype and treatment as factors, and the travelled distance prior to the shock-delivery as covariant, and this test was used to analyze the changes in mean velocity during the conditioning session as well. The locomotor activity preceding the delivery of shocks was analysed by two-way ANOVA [Factors: C21 effect and Genotype effect]. All ANOVA and MANOVA analyses were additionally analysed with Fisher's LSD. In contrast to contextual fear conditioning, all analyses of EPM were carried out with two-tailed unpaired t-test. The statistical significance was reached if the P value was below 0.05. The effect-sizes for both *in vitro* and *in vivo* treatments were determined by calculating Glass' delta values according to literature (Hedges and Olkin, 2014), and data was stored in FigShare under a CC-BY-SA license, DOI:10.6084/m9.figshare.12593396.

5 Results

5.1 C21 elevates the amount of sTRKB *in vitro*

Exposing cortical cells to different concentrations of C21 (0.1, 1.0, 10μM) for 15min led to the elevated amount of sTRKB [Kruskal-Wallis (3): 58.50, $P < 0.0001$; Figure 3A]. The concentration of sTRKB was increased in all groups treated with C21 (Dunn's, $P < 0.05$), from which the 10μM concentration appeared as the most effective one despite the larger distribution of the values [Glass' delta values: 0.1μM = 1.862; 1.0μM = 2.218; 10μM = 18.188]. Therefore, the 10μM concentration was used in the following *in vitro* experiments.

Since C21 appeared to have an impact on sTRKB, the effect of C21 on BDNF:TRKB signaling was studied next. As expected, a non-therapeutic treatment of BDNF (0.1 ng/ml) for 15min could not activate TRKB on its own, and similarly C21 (10μM) for 15min was also ineffective (Figure 3B). Interestingly, combining these treatments together successfully enhanced the effect of BDNF, inducing the activation of TRKB with a significant interaction between the treatments [Two-way ANOVA: C21 effect: $F(1, 33) = 3.755$, $P = 0.0613$; BDNF effect: $F(1, 33) =$

2.727, P = 0.1082; Interaction: F (1, 33) = 7.144, P = 0.0116]. Furthermore, when C21 treatment (10μM) was prolonged to three consecutive days, TRKB activation was significant in the C21-treated group (Figure 3C). In addition to this, a non-therapeutic treatment of BDNF (0.1 ng/ml) for 15min still elevated TRKB activation after pretreatment of C21 [Two-way ANOVA: C21 effect: F (1, 44) = 423.5, P < 0.0001; BDNF effect: F (1, 44) = 0.5861, P = 0.4480; Interaction: F (1, 44) = 10.22, P = 0.0026].

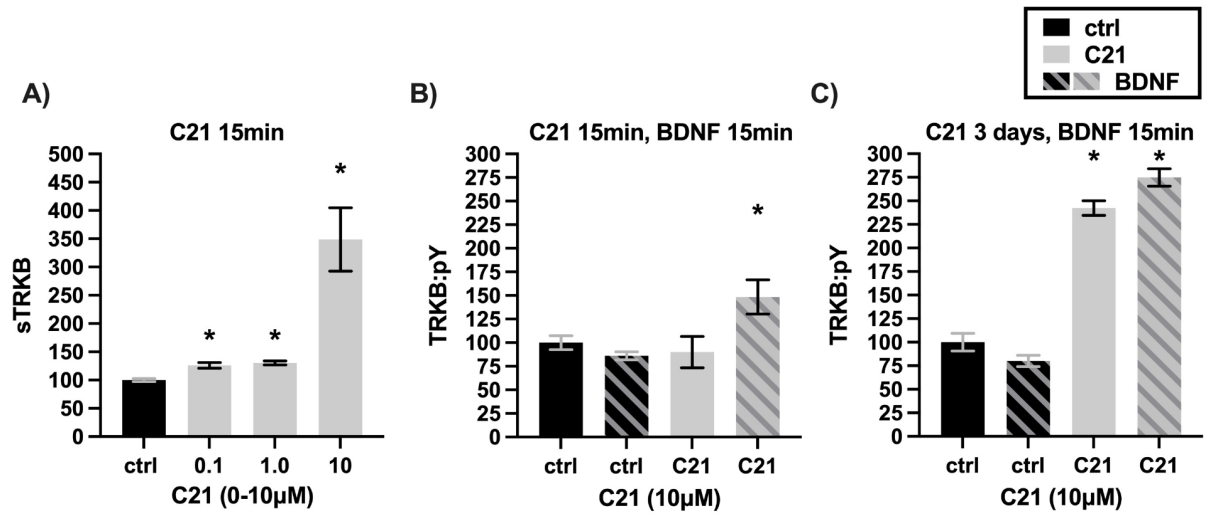

Figure 3. C21 effect on BDNF:TRKB signaling studied with ELISA. A) Exposing cells to C21 (0.1; 1.0; 10μM) for 15min significantly elevated the amount of sTRKB, 10μM concentration standing out as the most effective from the rest. **B)** Combination of C21 (10μM) for 15min and a non-therapeutic treatment of BDNF (0.1 ng/ml) for 15min activated TRKB. **C)** The effect on TRKB activation was further elevated in all cells predisposed to C21 (10μM) for three consecutive days. The cells that were additionally treated with BDNF (0.1 ng/ml) for 15min showed the strongest effect. Bars: mean/SEM of percentage compared to ctrl group of surface (sTRKB) and phosphorylated (TRKB:pY) TRKB. * p < 0.05 compared to ctrl.

5.2 C21 has an anti-stress effect in the contextual fear conditioning

The contextual fear conditioning was carried out following the N2B administered drug treatments (Figure 4), with four treatment groups: WT/vehicle, WT/C21, BDNF.het/vehicle and BDNF/C21. During the contextual fear conditioning that took place in Context A, three distinct electric shocks were delivered to all animals, which can be observed as the three peaks in mean velocity on each graph (Figures 5A - 5D). For clarity, the data for each group was plotted separately. The overall analysis of mean velocity during the conditioning session indicates no interaction between treatment, genotype and time [MANOVA: Interaction: F (14, 420) = 1.556, P = 0.089]. The between factors analysis indicates a significant interaction but no significant effect of treatment or genotype alone [MANOVA: C21 effect: F (1, 30) = 1.778, P = 0.192; Genotype effect: F (1, 30) = 0.0005, P = 0.981; Interaction: F (1, 30) = 7.056, P = 0.013].

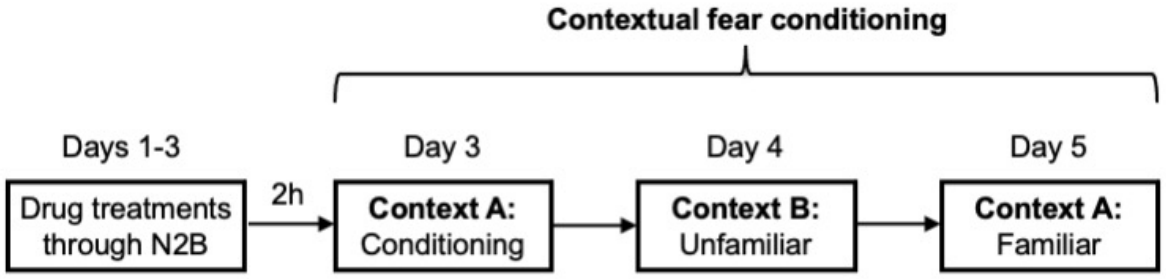

Figure 4. Timeline of contextual fear conditioning. Drug treatments: Before the actual experiment took place, all treatment groups (WT/vehicle, WT/C21, BDNF.het/vehicle, BDNF.het/C21) were treated with their assigned treatments for three consecutive days through a nose-to-brain route (N2B). The contextual fear conditioning consisted of three different phases that were carried out on different days as the timeline demonstrates: **Context A:** Conditioning; **Context B:** Unfamiliar and **Context A:** Familiar.

All WT mice showed similar levels of mean velocity regardless of their assigned treatment (Figure 5A - 5B). The first shock produced a weaker response in the BDNF.het/C21 group, which can be observed as a lower first peak of the mean velocity curve compared to the vehicle-treated animals of the same genotype (Fisher's LSD, $P < 0.0001$, Figure 4D). Despite that, all BDNF.het mice responded similarly to the following two shocks, indicating that this could be normal variation derived from the new context. As a conclusion, neither the treatment nor genotype affected the starting condition of the animals.

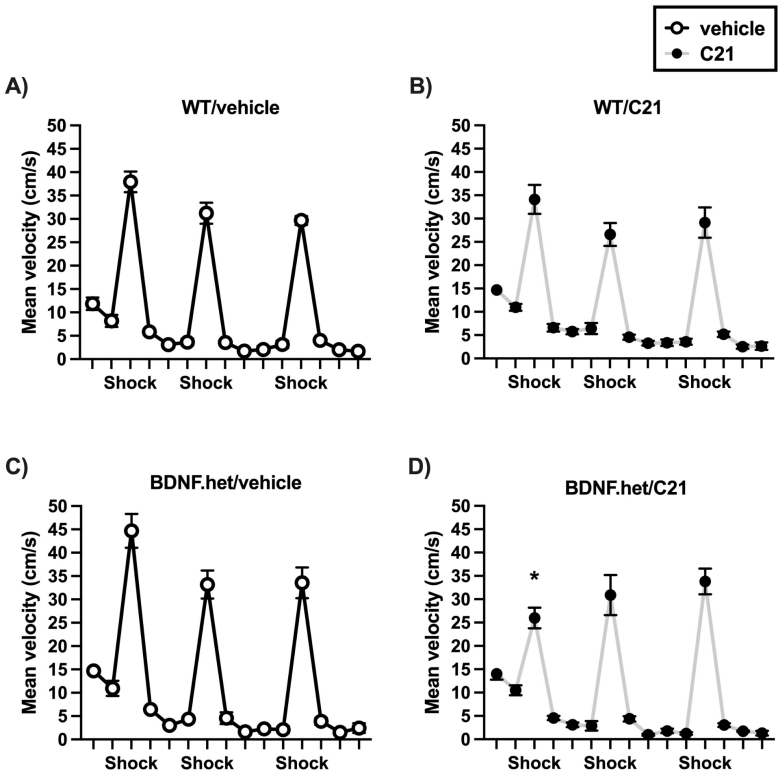

Figure 5. All animals received three shocks during the contextual fear conditioning. A-B) The graphs indicate how the mean velocity (cm/s) of WT mice increased in three different timepoints when the electric shocks were delivered during the fear conditioning (10min). As a conclusion, all mice received the shocks and there was no difference between the treatment groups (vehicle, C21). **C-D)** Similar to WT animals, both graphs indicate how three distinct electric shocks were delivered to BDNF.het mice in different timepoints during the fear conditioning (10min). BDNF.het/C21 mice had a weaker response to the first shock compared to the BDNF.het/vehicle mice. Graphs: mean/SEM of mean velocity (cm/s) along the conditioning session. *$p < 0.05$ compared to all other groups at the same time point.

The travelled distance, measured in the first 2min of the conditioning session (before the shocks), did not indicate any significant difference in the locomotion between WT and BDNF.het animals [Two-way ANOVA: C21 effect: $F(1, 30) = 1.078$, $P = 0.3074$; Genotype effect: $F(1, 30) = 1.178$, $P = 0.2864$; Interaction: $F(1, 30) = 2.629$, $P = 0.1154$; Figure 6A]. The travelled distance as covariant, the overall analysis of the freezing during the last 2min of the conditioning session, in the Context B and in the Context A, reveals a significant interaction between treatment, genotype and sessions [Two-way ANOVA, repeated measures: Interaction: $F(2, 58) = 3.631$, $P = 0.033$]. Therefore, in addition to overall analysis, each session was analysed separately. During the last 2min of the conditioning session, there was no difference in freezing between the groups regardless of their treatment or genotype [Two-way ANOVA: C21 effect: $F(1, 30) = 0.4826$, $P = 0.4926$; Genotype effect: $F(1, 30) = 0.6875$, $P = 0.4136$; Interaction: $F(1, 30) = 0.1451$, $P = 0.7059$; Figure 6B].

When the context was changed to the unfamiliar Context B, similar results were discovered in the terms of freezing as there was no interaction between the factors [Two-way ANOVA: C21 effect: F (1, 30) = 0.1342, P = 0.7167; Interaction: F (1, 30) = 0.03355, P = 0.8559; Figure 6C]. However, BDNF.het animals froze significantly more compared to WT animals, regardless of their treatment group [Two-way ANOVA: Genotype effect: F (1, 30) = 21.28, P < 0.0001]. Despite this, the results did not indicate that the animals would have been suffering from generalized fear after the conditioning session.

When animals were re-exposed to the familiar Context A, where they had received electric shocks during the fear conditioning, there was a significant interaction between the treatment and genotype [Two-way ANOVA: C21 effect: F (1, 30) = 18.34, P = 0.0002; Genotype effect: F (1, 30) = 55.60, P < 0.0001; Interaction: F (1, 30) = 5.012, P = 0.0327; Figure 6D]. Interestingly, the C21-treated WT mice froze significantly less compared to the WT/vehicle group (Fisher's LSD, p < 0.05; Glass' delta value = 2.847). However, BDNF.het/C21 animals did not benefit from the treatment (Glass' delta value = 0.613).

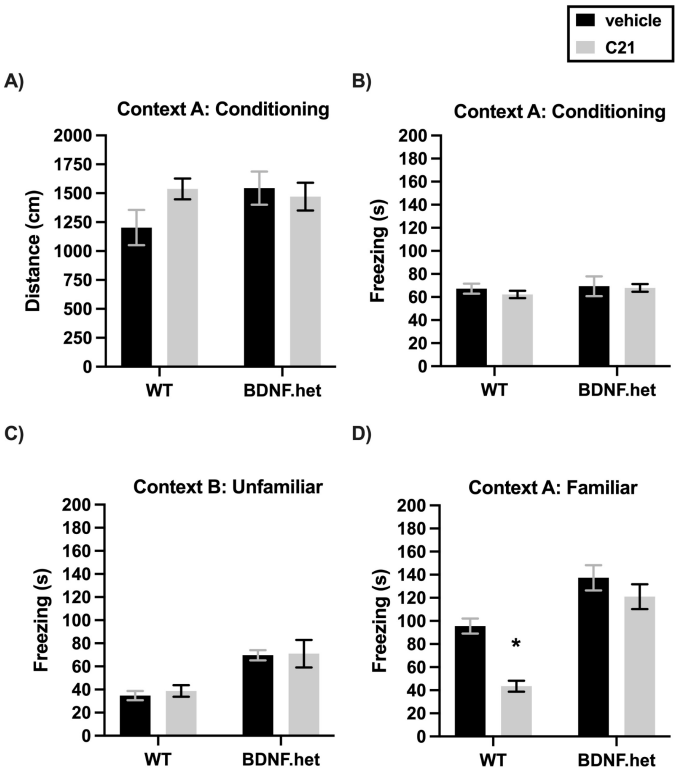

Figure 6. C21 protects WT mice from stress and this effect disappears in BDNF.het mice in the contextual fear conditioning. A) All animals regardless of their treatment group (WT/vehicle, WT/C21, BDNF.het/vehicle, BDNF.het/C21) showed similar amounts of activity during the first 2min of the contextual fear conditioning in the Context A (before the shocks). **B)** Furthermore, all animals froze as much during the last 2min in the Context A. **C)** In the unfamiliar Context B, animals did not freeze more, indicating that the conditioning did not induce generalized fear. Both BDNF.het groups froze more compared to the WT animals. **D)** In the familiar Context A, WT/C21 mice froze

significantly less. In contrast, BDNF.het animals showed overall more freezing and C21 treatment lacked effect in this genotype. Bars: mean/SEM of the freezing time (s). *p < 0.05 compared to the WT/vehicle group.

5.3 C21 did not relieve anxiety in the EPM

The anxiety-like behavior of animals was investigated with EPM. This was executed with another group of WT mice, specifically with WT/vehicle and WT/C21. Interestingly, the travelled distance was similar between the WT/vehicle and WT/C21 groups [Unpaired t-test: t = 0.03208; df = 10; P = 0,9750; Glass' delta value = 0.014; Figure 7A], and there was no difference in the EAE either [Unpaired t-test: t = 1.351, df = 10; P = 0,2064; Glass' delta value = 0.632; Figure 7B]. Similarly, both %OAE [Unpaired t-test: t = 1.097, df = 10; P = 0,2984; Glass' delta value = 0.592; Figure 7C] and %OAT [Unpaired t-test: t = 0.9135, df = 10, P = 0,3825; Glass' delta value = 0.525; Figure 7D] were not affected by the administration of C21.

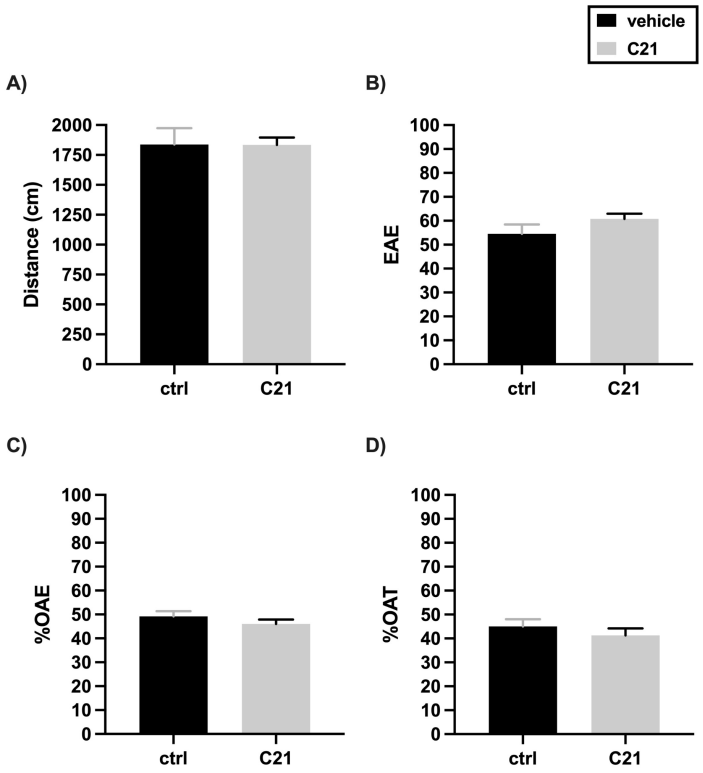

Figure 7. C21-treated WT mice indicated as much anxiety as the vehicle-treated animals in the EPM. A) There was no difference between the activity of WT/vehicle or WT/C21 mice. The activity was measured as travelled distance (cm) in 5min. **B)** WT/vehicle mice showed as much willingness to enter the enclosed arms of the plus-maze as the WT/C21 mice, measured as entries in the enclosed arms (EAE). **C)** Likewise, the percentage of open arm entries (%OAE) was similar between the WT/vehicle and WT/C21 mice, indicating that C21 did not induce any anxiolytic effect in mice. **D)** Supporting this, the open arm time (%OAT) of the plus-maze was similar in the WT/C21 group. Bars: mean/SEM.

5.4 AGTR2 is expressed in the GABAergic interneurons

In order to have insights about what cells express AGTR2, the levels of this protein were mined *in silico* from the Allen Institute database. Since the AGTR2 expression was only significant in the GABAergic cells (top row), only this class was plotted in the heatmap (Figure 8). The heatmap shows that compared to other cell subclasses, AGTR2 expression seems to overlap with *Lamp5* and *Sst* expressing cells, from which the expression is stronger in the *Sst* subtype. Compared to *Pvalb* positive (PV+) interneurons, another subclass of GABAergic interneurons, the expression of AGTR2 in the *Sst* positive (SST+) interneurons is clearly stronger. These results indicate that AGTR2 are mostly located in the subclass of GABAergic, in *Lamp5* positive (Lamp5+) and in SST+ interneurons.

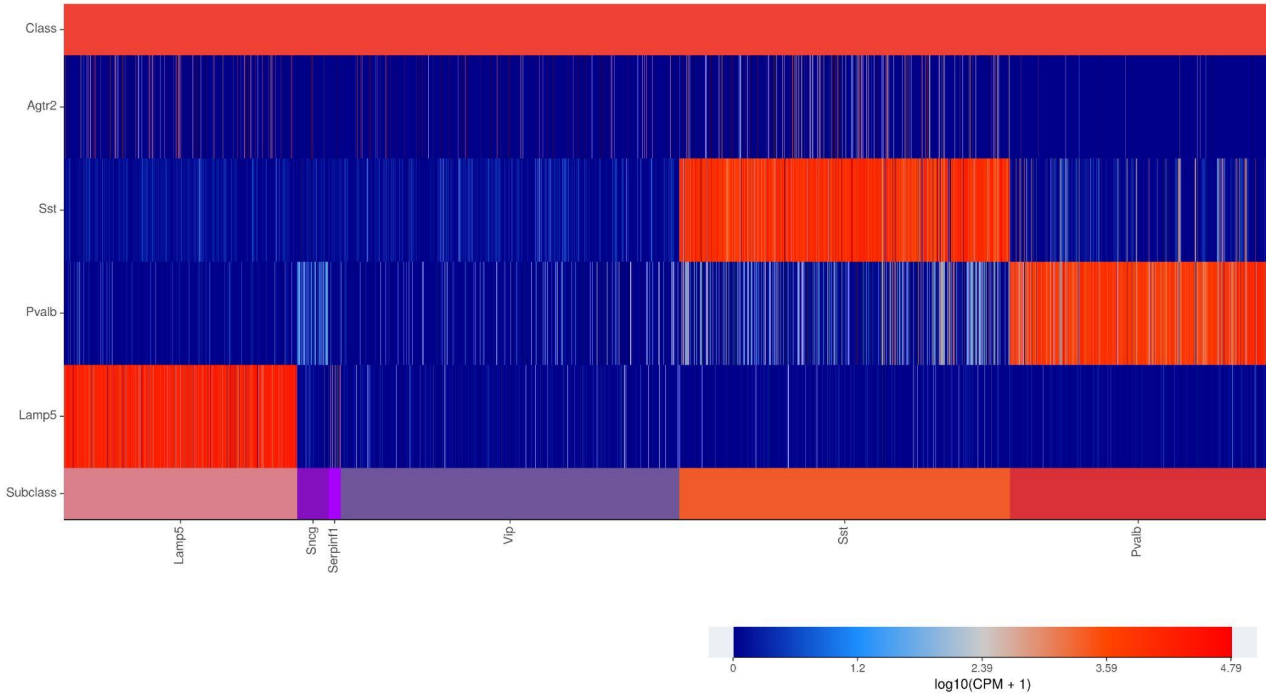

Figure 8. Heatmap of AGTR2 expression in different GABAergic neuronal subtypes. Cell class: GABAergic = top row. **Cell subclasses:** *Lamp5, Sncg, Serpinf1, Vip, Sst, Pvalb*. The expression of AGTR2, presented as vertical lines in a logarithmic scale. The amount of expression is presented as a spectrum of colors from blue to red, blue being smaller and red being stronger. Some of the subclasses that seemed to express more AGTR2 were observed in more detail. The heatmap shows that AGTR2 expression is linked to the GABAergic subclass of SST+ interneurons. Allen Mouse Brain Atlas (Lein et al., 2007; Morris et al., 2010) was used for data mining.

6 Discussion

This study shows that AGTR2 stimulation by different concentrations of C21 for 15min results in elevated amounts of sTRKB in cortical cell cultures. A similar effect has been observed with ANG through an AGTR2-dependent mechanism, where heterodimerization of TRKB:AGTR2 was also present (Diniz et al., 2018). Even though TRKB is located in the cell membrane, it is mostly found inside vesicles within the cells where it is being driven into the cell membrane following BDNF:TRKB signaling to allow more interaction (Haapasalo et al., 2002). Furthermore, ADs have been shown to compromise the clathrin-dependent endocytosis that keeps the TRKB internalized, and thus increase the localization of TRKB in the surface, which has been suggested to play a crucial role in their mechanism of action (Fred et al., 2019). Results of the present study suggest that the therapeutic effect of C21 might work through a similar mechanism, despite failing to activate TRKB after acute administration. Interestingly, the prolonged treatment of C21 achieved this effect. This could be a consequence of the elevated amount of sTRKB that might enhance BDNF:TRKB signaling after a prolonged AGTR2 stimulation.

Furthermore, AGTR2 stimulation by C21 and an additional treatment of non-therapeutic BDNF both acutely and after a prolonged trial were followed by TRKB activation. As known, TRKB plays a crucial role in the mechanism of action of ADs (Castrén and Antila, 2017) and thus, allowing more TRKB to access the cell surface facilitates its interaction with BDNF and consequently BDNF:TRKB signaling. Hence, the elevated amount of sTRKB that follows the AGTR2 stimulation seems to be behind the facilitation of BDNF-induced activation of this receptor. Furthermore, according to our recent study the stabilization of the signaling-competent conformation of TRKB is behind the mechanism of several different ADs (Casarotto et al., 2021).

According to the results of the contextual fear conditioning AGTR2 stimulation protects from consequences of stress. The animals pretreated with C21 exhibited lower freezing when re-exposed to the familiar context that had been associated with the fear-inducing stimulus during conditioning. Moreover, this therapeutic effect on stress was completely absent in BDNF.het mice. Similarly, the BDNF.het animals were resistant to the antidepressant-like effect that relieved the consequences of stress in WT animals in the unpredictable chronic mild stress (UCMS) paradigm (Ibarguen-Vargas et al., 2009), and these animals also resisted the effects of AGTR1 antagonist losartan in the FST (Diniz et al., 2018). In the present study BDNF was also necessary for elevated activation of TRKB *in vitro*, and thus the lack of effect *in vivo* points to the same conclusion that

the therapeutic effects of C21 are dependent on BDNF and possibly a consequence of elevated sTRKB.

Interestingly, the results of EPM indicate that C21 does not work as an anxiolytic, which is surprising, since deficiency of AGTR2 has been associated with increased anxiety (Okuyama et al., 1999). Since pretreatment with C21 was able to protect from the consequences of stress in the contextual fear conditioning, this could indicate an important confounding effect at the conditioning session and responsiveness to stress. It is widely accepted that glutamate is a crucial regulator of our stress response as it indirectly elevates the amount of glucocorticoids in response to acute and prolonged stress through the stimulation of hypothalamus-pituitary-adrenal (HPA) axis (Evanson and Herman, 2015). When paraventricular nucleus (PVN) of the hypothalamus is stimulated by glutamate, corticotropin-releasing hormone (CRH) secretion is triggered, which leads to adrenocorticotropic hormone (ACTH) release from anterior pituitary, and finally the production of glucocorticoids in the adrenals (Evanson and Herman, 2015). Moreover, the synthesis of glucocorticoid receptors located in the prefrontal cortex (PFC) has been shown to diminish in response to chronic stress (Chiba et al., 2012). Of importance, several AGTR1 antagonists have been shown to prevent the stress-induced elevation of glucocorticoids (Pavel et al., 2008; Wincewicz et al., 2016; Campos et al., 2020). Furthermore, exposing cortical cells to glutamate for 24h shifts the ratio between AGTR1 and AGTR2 synthesis towards AGTR1, preventing ANG from activating TRKB through AGTR2 without previous AGTR1 blockade by losartan (Diniz et al., 2018). Stress has been shown to decrease BDNF synthesis in the mPFC, which can be prevented by blocking AGTR1 with telmisartan (Wincewicz et al., 2016). Stress is also followed by reduced synthesis of corticotropin-releasing factor 1 (CRF1) in the cortex, again prevented by an AGTR1 blockade (Saavedra et al., 2006; Pavel et al., 2008).

Similar to present study with C21, AGTR1 antagonists candesartan and telmisartan did not induce any anxiolytic effect in the EPM whereas they still alleviated the stress-induced deficits on the cognitive function (Braszko et al., 2013; Wincewicz and Braszko, 2014). However, there are some contradictory studies in literature where different AGTR1 antagonist treatments are able to alleviate anxiety in the EPM (Saavedra et al., 2006; Pavel et al., 2008; Wincewicz et al., 2016). For example, AGTR1 blockade by losartan was followed by an anxiolytic-like effect in the EPM even though this treatment was not able to attenuate the elevated AGTR1 synthesis in the HC of these animals (Campos et al., 2020). Taken together, several pieces of evidence suggest that the blockade of AGTR1 seems to indirectly enhance AGTR2 function, which in turn engages BDNF:TRKB system to exert its effects (Diniz et al., 2018). This indirect activation of AGTR2 could explain how

these compounds attenuate the stress-induced increase in the glutamate level, glucocorticoids and increased AGTR1 activity.

On the other hand, the AGTR2-mediated effect could also be dependent on the cell type that expresses these receptors and the exact location of these cells in the brain, especially involving the fear-regulating circuits. Direct infusion of losartan into the HC and mPFC of rats induced an antidepressant-like effect in the FST, indicating a significant role of these two brain regions in the therapeutic effects of AGTR2 stimulation (Diniz et al., 2018). However, when this effect was investigated in more detail by inducing the AGTR2 blockade in both structures, the effect of systemic losartan was only compromised by previous AGTR2 blockade in the mPFC (Diniz et al., 2018). As stated previously, the mPFC is crucially involved in the regulation of both conditioned and unconditioned fear and, in fact, AGTR2 is expressed in neurons located in the mPFC, with low expression in the hippocampus (de Kloet et al., 2016). Furthermore, some of these neurons are located in the amygdala and BNST, the latter of which were further speculated to send GABAergic projections to the PVN that by itself did not express AGTR2 (de Kloet et al., 2016).

Using the Allen Mouse Brain Atlas to mine for information about the neuronal subtypes expressing AGTR2, we observed that AGTR2 expression significantly overlaps with GABAergic SST+ interneurons. In contrast, the AGTR2 expression in the PV+ interneurons is almost non-existent. This and the role of SST+ interneuron populations in the mPFC, amygdala and BNST suggests that SST+ interneurons located in these brain regions might be responsible for the therapeutic effects of C21. MDD has been linked to a significant attenuation of the SST synthesis in the PFC (Sibille et al., 2011). In addition, SST seems to possess mood recovering effects as a direct infusion into the cerebrospinal fluid has been associated with attenuated anxiety- and depressive-like behavior in rats (Engin et al., 2008). Furthermore, it has been demonstrated that a chronic exposure to stress induces plastical changes in the mPFC that results in the elevated synthesis of GABA A α1 (GABAAα1) receptor and morphological changes such as dendritic hypertrophy especially in the GABAergic SST+ interneurons (Gilabert-Juan et al., 2013).

After the exposure to chronic stress, the synthesis of SST has been shown to diminish in the mPFC located SST+ interneurons in mice (Jefferson et al., 2020). However, the disinhibition of SST+ interneurons protects from this deficit, and additionally alleviates other consequences of stress in male mice only (Jefferson et al., 2020). This sex-specific finding also supports the results of a previous clinical study, where the MDD was linked with attenuated synthesis of subgenual anterior cingulate cortex (sACC)-located SST especially in females (Tripp et al., 2011). One of the limitations of the present study is that the effects of C21 were only investigated in the female

animals. Therefore, possible sex-specific effects remain unknown. Even though the animals responded to the treatment in the contextual fear conditioning, a sex-specific effect might partly explain the results of EPM. However, this remains only as a speculation until studied further.

Disinhibition of forebrain-located SST+ interneurons elevates their inhibitory effect on HC-located principal neurons, which is followed by behavioral improvement of mice in the different paradigms measuring stress and anxiety (Fuchs et al., 2017). Similarly, disinhibition of amygdala located SST+ interneurons and the following attenuation in the activity of principal neurons seem to compromise fear conditioning (Wolff et al., 2014). This study indicated that the conditioned fear is a consequence of principal neuron disinhibition, which is linked to the elevated activity of PV+ interneurons that reduce the inhibitory signaling of SST+ interneurons (Wolff et al., 2014). Furthermore, the unconditioned fear was associated with attenuated activity of both SST+ and PV+ interneurons, the latter of which was responsible for observed disinhibition of principal neurons (Wolff et al., 2014).

These findings are interesting, especially considering the results of the present study where the therapeutic effects of C21 were only present in the contextual fear conditioning but not in the EPM. As AGTR2 is expressed in the SST+ interneurons, the AGTR2 stimulation by C21 might be able to recruit these interneurons to enhance their GABAergic signaling on principal neurons in PFC and amygdala, and thereby attenuate conditioned fear. Even though the activity of PV+ interneurons seems to regulate the activity of SST+ interneurons, C21-induced AGTR2 activity in SST+ interneurons might be able to oppose their inhibitory effect. Furthermore, the lack of effect in the EPM could be explained by the PV+ interneurons that at the same time seem to regulate unconditioned fear and do not express AGTR2. This would also support the idea that the conditioning session and exposure to stress play a crucial role in the therapeutic effect of C21, suggesting that C21 is able to protect from the conditioned fear specifically.

In contrast to other studies, the mPFC-located SST+ interneurons have been suggested to regulate conditioned fear and indicate that inhibiting their GABAergic function reduces the consequences of conditioned fear (Xu et al., 2019; Cummings and Clem, 2020). According to these studies, the mPFC-located SST+ interneurons inhibit the activity of PV+ interneurons, thus reducing their GABAergic signaling to their target neurons, which indirectly leads to disinhibition of the principal neurons (Xu et al., 2019; Cummings and Clem, 2020). In line with other studies, the activity of mPFC and disinhibition of principal neurons were crucially involved in the mechanism that induced conditioned fear according to these studies (Xu et al., 2019; Cummings and Clem, 2020). This raises the question whether SST+ interneurons act differently depending on

their location in the brain. Since the studies conducted in the forebrain and mPFC resulted in entirely opposite results, the SST+ and PV+ interneurons should be studied more to confirm how they are involved in the regulation of fear.

Furthermore, it is rather likely that different brain regions are also involved in the mechanism of action of C21, especially since AGTR2 expressing cells can be also found from the BNST. Taking this together with the knowledge of fear circuits, it might be possible that C21-induced AGTR2 activity in the mPFC and amygdala located SST+ interneurons that could further signal to BNST and indirectly attenuate the activity of PVN and entire HPA axis. However, this remains under speculation as long as the exact cell type regulating the effect of C21 remains unidentified. The future studies should focus on answering this question to widen the current knowledge of how the therapeutic effects of C21 are generated. Since C21 can be easily administered through N2B, this might make it easier to translate this drug into clinical use for treating mood disorders in the future.

7 Conclusions

According to both *in vitro* and *in vivo* results of this study the BDNF:TRKB signaling plays a crucial role in the mechanism of action of C21. Stimulation of AGTR2 by C21 allows TRKB to access the cell surface in larger amounts, followed by enhanced BDNF:TRKB interaction and the receptor activation. Nevertheless, by itself C21 fails to activate TRKB. Therefore, AGTR2 stimulation induces a facilitatory effect on BDNF.

C21 has stress-protective effects where the AGTR2 stimulation by this compound attenuates the contextual fear conditioning -induced stress without relieving anxiety in the elevated plus-maze, thus indicating the dependence of the exposure to stress. The essential role of BDNF in the therapeutic effect of C21 is apparent as it disappears in the BDNF-deficient animals. The C21 effect may be linked to the SST+ interneurons in the mPFC but future studies are needed to test this hypothesis, and to further develop AGTR2 agonist C21 as a viable therapeutic approach to treat neuropsychiatric disorders.

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